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fldA is an essential gene required in the 2-*C*-methyl-D-erythritol 4-phosphate pathway for isoprenoid biosynthesis

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Abstract Although flavodoxin I is indispensable for *Escherichia coli* growth, the exact pathway(s) where flavodoxin I is essential has not been identified. We performed transposon mutagenesis of the flavodoxin I gene, *fldA*, in an *E. coli* strain that expressed mevalonate pathway enzymes and that had a point mutation in the *lytB* gene of the MEP pathway resulting in the accumulation of (*E*)-4-hydroxy-3-methylbutyl-2-enyl pyrophosphate (HMBPP). Disruption of *fldA* abrogated mevalonate-independent growth and dramatically decreased HMBPP levels. The *fldA*$^-$ mutant grew with mevalonate indicating that the essential role of flavodoxin I under aerobic conditions is in the MEP pathway. Growth was restored by *fldA* complementation. Since GcpE (which synthesizes HMBPP) and LytB are iron–sulfur enzymes that require a reducing system for their activity, we propose that flavodoxin is essential for GcpE and possibly LytB activity. Thus, the essential role for flavodoxin I in *E. coli* is in the MEP pathway for isoprenoid biosynthesis.

Keywords: 2-*C*-Methyl-D-erythritol 4-phosphate; Flavodoxin I; *fldA*; (*E*)-4-Hydroxy-3-methylbutyl-2-enyl pyrophosphate; γδ T cell

1. Introduction

Two metabolic pathways exist for isoprenoid biosynthesis: the classical mevalonate pathway found ubiquitously in mammalian cells and Archaebacteria and the recently discovered 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway (also termed the deoxyxylulose pathway) [1–3]. The MEP pathway is present in most *Eubacteria*, plant chloroplasts, and the apicoplasts of Apicomplexan protozoa such as *Plasmodium* and *Toxoplasma* [4]. Both pathways lead to the synthesis of two essential metabolic precursors for isoprenoid biosynthesis, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). The MEP pathway consists of 7 enzymes. The last two enzymes, GcpE [5–7] and LytB [8], are the most unusual because their functional activities are rapidly inactivated by dioxygen and thus are dependent on the presence of exogenous reducing agents for their enzymatic activity (reviewed in [4]). Seemann et al. [9] has reported that GcpE in *E. coli* consists of a dioxygen-sensitive $[4Fe–4S]^{2+}$ cluster that is required for the catalytic reduction of 2-*C*-methyl-D-erythritol cyclopyrophosphate into (*E*)-4-hydroxy-3-methylbutyl-2-enyl pyrophosphate (HMBPP).

HMBPP is a potent activator of human Vγ2Vδ2 T cells ([10,11], and reviewed in [12]) and the presence of HMBPP correlates with the ability of microbes to stimulate Vγ2Vδ2 T cells in vitro and in vivo ([13], and reviewed in [14]). Similarly, the conversion of HMBPP to IPP and DMAPP by the downstream enzyme, LytB, also strictly depends on the presence of reducing agents for its action [15]. Further studies on LytB using UV/Vis absorption and electron paramagnetic resonance reveal that it too contains a [4Fe–4S] cluster [16,17]. Thus, it has been suggested that these [Fe–S] clusters in GcpE and LytB participate in the electron transfer process maintained under a reducing environment.

A common characteristic shared by many iron–sulfur proteins is the requirement for a reducing system that permits the regeneration of reduced $[Fe–S]^+$ clusters. The reduction of oxidized $[Fe–S]^{2+}$ could be achieved using dithionite or a stronger reductant, deazaflavin [15]. Alternatively, this reduction can be provided using the endogenous flavodoxin/flavodoxin reductase/NADPH reducing system present in prokaryotes, and in red and green algae. Flavodoxins comprise a family of small molecular weight electron transferases important for a variety for metabolic processes including nitrogen fixation, carbon dioxide fixation, and anaerobic metabolism. Two types of flavodoxins exist in *E. coli*. Flavodoxin I is encoded by *fldA* and is constitutively expressed in *E. coli* whereas flavodoxin II is encoded by *fldB* and is induced by oxidative stress [18]. Flavodoxin I has been shown to be an essential gene in *E. coli*. Flavodoxin II cannot replace flavodoxin I [18]. However, the metabolic pathway where flavodoxin I is essential is unknown. Here, we report that transposon-mediated mutagenesis of *fldA* demonstrates that flavodoxin I plays an essential role in the MEP isoprenoid biosynthetic pathway.

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Abbreviations: DMAPP, dimethylallyl pyrophosphate; IPP, isopentenyl pyrophosphate; MEP, 2-*C*-methyl-D-erythritol 4-phosphate; HMBPP, (*E*)-4-hydroxy-3-methylbutyl-2-enyl pyrophosphate; LB, Luria-Bertani; MVA, mevalonate; PBS, phosphate buffered saline

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2. Materials and methods

2.1. Construction of the DK310 idi-disruptant

Bacteria strains and plasmids used in this study are detailed in Supplemental Table 1. On the basis of the nucleotide sequence of the IPP isomerase gene, *idi*, from *E. coli* W3110, two oligonucleotide primers, 5′-GGAAGCTTACATGATGCTTCATGGTTTG-3′ (upstream of 5′ of idi) and 5′-GGAAGCTTTGGGAAGTTGAAACGCCGATG-3′ (downstream of 3′ of idi), including a *Hin*dIII restriction site (underlined) were synthesized (Sigma, St. Louis, MO) and used together with total DNA from *E. coli* W3110 to amplify *idi*. By using Taq DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN) and the protocol recommended by the supplier, a 2.5-kb fragment including the *idi* gene was amplified. The PCR fragment was digested with *Hin*dIII and cloned into the multi-cloning site of an *E. coli* vector pUC118 to give pUIDI.

An *idi*-disruptant, DK310, was constructed by insertion of the Cm^r gene (a chloramphenicol-resistance gene) into the *idi* gene. Cm^r gene was digested with *Aor*51HI from pACYC184 to provide a 1.3-kb DNA fragment carrying the Cm^r gene, which encoded chloramphenicol acetyltransferase. pUIDI was digested with *Spe*I, the recognition site of which was in the targeted *idi* gene, treated with T4 DNA polymerase, and then ligated with the 1.3-kb DNA fragment carrying the Cm^r gene to construct pUIDICM. A 3.8-kb linear *Hin*dIII–*Hin*dIII fragment carrying *idi* and Cm^r genes was isolated from pUIDICM. *E. coli* FS1576, a *recD* mutant, was transformed with the *Hin*dIII–*Hin*dIII fragment and then chloramphenicol-resistant transformants were selected on Luria-Bertani (LB) plates containing 17 μg chloramphenicol/ml. After the correct disruption was confirmed by Southern hybridization using the *idi* and Cm^r genes as the probes, the disrupted *idi* gene was then transduced to *E. coli* W3110 (wild-type) using bacteriophage P1. The resulting W3110 *idi*::Cm^r strain was named DK310.

2.2. Construction of DK310 (idi^-) $LytB^{G120D}$ mutant strains

The idi disruptant strain, DK310, was treated with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine as previously described [19]. After the treatment, DK310 was transformed with the pTMV22KM plasmid that contains the genes for mevalonate kinase, phosphomevalonate kinase, and diphosphomevalonate decarboxylase all from *Streptomyces* sp. strain CL190 [20,21]. A mutant in the *lytB* gene was then isolated by screening ~20000 colonies for colonies that required mevalonate for growth. The point mutation changed glycine to aspartic acid at position 120 (GGT → GAT). This strain was named DK310 $LytB^{G120D}$ (pTMV22KM). A second strain, DK310 $LytB^{G120D}$ (pTMV20KM), was developed from this strain by transformation with pTMV20KM and selection on an LB plate containing CM and KM. The pTMV20KM plasmid contains the *Streptomyces* sp. genes for mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, and isopentenyl diphosphate isomerase. The growth properties of the two strains at 37 °C in LB liquid medium for 12 h are shown in Table 1.

2.3. Generation of DK310 $LytB^{G120D}$ fldA mutant strain

An *fldA* mutant *E. coli* strain was generated by transposon-mediated mutagenesis of the DK310 $LytB^{G120D}$ (pTMV20KM) bacteria. The bacteria were grown in LB broth to an optimal density of 0.3, chilled immediately, centrifuged, and washed three times with ice-cold 10% glycerol. The resulting pellet was re-suspended in medium containing 10% glycerol, 0.125% (w/v) yeast extract and 0.25% (w/v) tryptone, and stored at −80 °C. Fifty microliter of these electrocompetent bacteria was mixed with 1 μl of EZ:TN™ ⟨DHFR-1⟩ Tnp Transposome™ (Epicentre, Madison, WI) and transferred to a 0.2 cm electrode curvette. Transposome were electroporated into bacterial cells using a Gene Pulser II electroporator (Biorad, Burlingame, CA) set at 2500 V, 200 Ω, and 25 μF. One milliliter of SOC medium was immediately added to the bacteria after electroporation. The bacteria were incubated at 37 °C for 1 h with gentle agitation. The transposed bacteria were spread on LB plate containing 50 μg/ml trimethoprim and 1 mg/ml mevalonolactone (Sigma) at 37 °C overnight. Bacterial mutants, thus, generated were arrayed in a 96 well format and screened for the loss of bioactivity with a Vγ2Vδ2 T cell clone and for their ability to grow in the absence of mevalonate. To ensure the complete loss of activity, bacteria were further grown at room temperature for 4–7 days. Genomic DNA was isolated from mutants using a MasterPure™ DNA purification kit (Epicentre) and directly sequenced with a pair of primers specific to each end of the transposon at the University of Iowa DNA sequencing facility. DHFR-1 forward primer: GGCGGAAACATTGGATGCGG; DHFR-2 reverse primer: GACACTCTGTTATTACAAATCG. The genomic transposition sites were located using BLAST programs maintained at the NCBI web site of the National Library of Medicine (http://www.ncbi.nlm.nih.gov/BLAST/). The *fldA* mutant had a transposon inserted into the *fldA* coding sequence at nucleotide 39.

Table 1
Growth properties of the DK310 $LytB^{G120D}$ mutants[a]

Growth condition	DK310 $LytB^{G120D}$ (pTMV22KM)	DK310 $LytB^{G120D}$ (pTMV20KM)
CM[b] and KM[c]	–	++
CM, KM, and MVA[d]	++	++
CM, KM, and FMM[e]	–	–
CM, KM, MVA, and FMM	–	+

[a]Bacteria were grown in LB liquid medium for 12 h at 37 °C.
[b]Chloramphenicol was present at 34 μg/ml.
[c]Kanamycin was present at 25 μg/ml.
[d]Mevalonolactone was present at 0.01%.
[e]Fosmidomycin was present at 12.5 μg/ml.

2.4. Complementation of the fldA mutant

To complement the DK310 $LytB^{G120D}$ $fldA^-$ (pTMV20KM) mutant, the *fldA* gene was cloned from *E. coli* into the low-copy vector, pMW118, at the *Bam*HI site to derive the plasmid, pMW-*fldA*. The insert, containing the upstream sequence and the full coding sequence of the *fldA* gene, is given in Supplemental Figure 1. The DK310 $LytB^{G120D}$ $fldA^-$ (pTMV20KM) mutant was transformed with the pMW-*fldA* plasmid by electroporation as described above. Transformants were selected by resistance to ampicillin and trimethoprim and then tested for their growth with and without mevalonate.

2.5. Preparing bacterial supernatants and sonicates

To test bacterial supernatants and sonicates for their ability to stimulate Vγ2Vδ2 T cells, *E. coli* bacteria were grown in 1 L of LB media in 2.6 L fluted Fernback flasks to late stationary phase by incubating for ~24 h at 37 °C in an Innova 4400 shaker oscillating at 225 rev/min. This was found to result in the maximum amount of bioactivity. The bacteria were harvested by centrifugation at $380 \times g$ for 15 min at 4 °C. The culture supernatant was removed and the bacteria washed twice with phosphate buffered saline (PBS). Bacteria from 1 L of culture were suspended in 10 ml of PBS and continuously probe sonicated for 10 min on ice at a 4.5 setting (Sonic Dismembrator Model 550, Fisher Scientific). The sonicated bacteria were centrifuged at $300 \times g$ for 15 min at 4 °C. The supernatants from the sonicated bacteria and the culture supernatants were heated in a boiling water bath for 5 min, cooled on ice for 5 min, centrifuged at $16000 \times g$ for 30 min at 4 °C, filter sterilized with a 0.22 μm filter, and frozen at −80 °C. The heating caused precipitation of protein and other bacterial components that inhibit T cell proliferation but did not affect the overall bioactivity for Vγ2Vδ2 T cells.

2.6. Assaying bioactivity for Vγ2Vγ2 T cells

The 12G12, DG.SF68, and CP.1.15 Vγ2Vδ2 T cell clones used in this study have been described [22]. Vγ2Vδ2 T cell clones were propagated by periodic re-stimulation as described [23]. Briefly, $2.5–5 \times 10^5$ T cells were cultured with irradiated PBMC (2.5×10^5) from healthy donors, EBV-transformed B cells (DG.EBV and CP.EBV each at 2.5×10^5) and PHA (1:2000) in RPMI 1640 supplemented with rIL-2 (1 nM).

For assaying bioactivity, $5–10 \times 10^4$ Vγ2Vδ2 T cells were cultured with $5–10 \times 10^4$ mitomycin C-treated Va-2 cells (a transformed human fibrosarcoma cell line) as antigen presenting cells in 200 μl of complete medium (RPMI 1640 with 8% FBS, L-glutamine, sodium pyruvate, non-essential, essential MEM amino acids, HEPES, and β-mercaptoethanol). Culture supernatants and bacterial sonicates were added to the cultures at half-log dilutions and incubated at 37 °C. The cultures were pulsed with 1 μCi of ^{3}H-thymidine after 24 h and harvested 16–24 h later.

To quantitate bioactivity, the dilution of the bacterial supernatant or sonicate that gave half-maximal proliferation was determined. The reciprocal of the dilution gave the units of bioactivity. Therefore, one unit of bioactivity was the antigen amount in 1 ml that gave half-maximal antigen-induced proliferation of a Vγ2Vδ2 T cell clone. This corresponds to an HMBPP concentration of 31.6 pM or 31.6 fmol/ml and an IPP concentration of 3 μM or 3 nmol/ml. To control for variations in this assay (about 3-fold), a standard preparation of ethylpyrophosphate that contained 12000 U/ml was assayed with each experiment. Bioactivity values were adjusted relative to this standard. Total bioactivity for a 1 L culture was the sum of the bioactivity present in the bacterial sonicates and culture supernatants. For stationary phase and late stationary phase cultures of wild-type *E. coli*, 77% and 95% of the bioactivity was in the supernatant, respectively. In the DK310 LytB^{G120D} (pTMV20KM) and DK310 LytB^{G120D} *fldA*$^{-}$ (pTMV20KM), 91% and 43% of the bioactivity was in the supernatant, respectively.

3. Results and discussion

3.1. Substitution of aspartic acid for glycine at position 120 in LytB in DK310 results in the loss of IPP but not DMAPP synthesis

A point mutation was made in the *lytB* gene of DK310 pTMV22KM that lacks isopentenyl diphosphate isomerase by chemical mutagenesis such that aspartic acid was substituted for glycine at residue 120. This residue is in an area that is highly conserved between *E. coli*, *Mycobacterium tuberculosis*, the cyanobacteria, *Synechocystis*, the plant, *Adonis aestivalis*, and the apicomplexan parasite, *Plasmodium falciparum* and near an additional area of conservation around the cysteine residue 96 [4,24]. The mutant, DK310 LytB^{G120D} pTMV22KM (*idi*$^{-}$), can synthesize IPP in the presence of mevalonate through the introduced mevalonate pathway enzymes and is mevalonate dependent (Table 1). Since the mutant lacks isopentenyl diphosphate isomerase, it cannot isomerize IPP to DMAPP. This is the only way to interconvert IPP and DMAPP in *E. coli* [25]. Based on this fact, we hypothesize that the LytB^{G120D} mutant must continue to synthesize DMAPP but has lost the ability to synthesize IPP.

Consistent with this hypothesis, blocking the MEP pathway with fosmidomycin prevented bacterial growth (Table 1). Moreover, reintroduction of isopentenyl diphosphate isomerase from *Streptomyces* sp. in DK310 LytB^{G120D} pTMV20KM (Table 1) restored mevalonate-independent growth and fosmidomycin resistance. Since DMAPP synthesized by LytB could isomerize to IPP and IPP synthesized by phosphomevalonate decarboxylase could isomerize to DMAPP, bacterial growth occurred even when each individual pathway was blocked (see Fig. 1).

3.2. Flavodoxin I is essential for isoprenoid biosynthesis

To examine genes that are involved in isoprenoid biosynthesis, the DK310 LytB^{G120D} pTMV20KM strain was subjected to random transposon mutagenesis. Mevalonate was introduced to rescue bacteria since mutations in the MEP pathway are lethal in its absence. A total of 27 mutants were selected for their failure to grow independently of MVA. Direct genome sequencing of these mutants reveals that 25 had transposon insertions in genes of enzymes within the MEP pathway. One gene not previously reported to be essential in the MEP pathway was *fldA*. Mutation of *fldA*, as with mutation of all the genes present in the MEP pathway, resulted in the failure

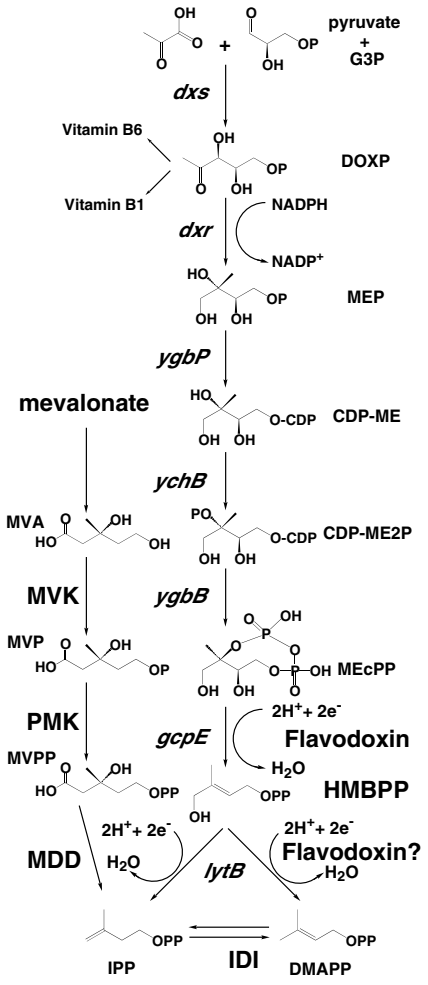

Fig. 1. The MEP (deoxyxylulose) pathway of isoprenoid biosynthesis and the engineered biosynthesis of IPP from exogenous mevalonate [1–4]. Proposed steps of flavodoxin action at GcpE and possibly at LytB are shown. MEP enzymes are also termed IspC (Dxr), IspD (YgbP), IspE (YchB), IspF (YgbB), IspG (GcpE), and IspH (LytB). The DK310 bacteria were transformed with a plasmid containing the genes for the following mevalonate pathway enzymes: MVK, mevalonate kinase; PMK, phosphomevalonate kinase; MDD, mevalonate diphosphate decarboxylase.

of the mutant to grow on LB agar unless exogenous mevalonate was provided (Fig. 2B and C).

Since the functional activity of GcpE [7,15] and LytB [8,16,17,26] depends on the availability of reduced $[4Fe–4S]^{+}$ clusters, a defect in flavodoxin I function appears to block the regeneration of reduced clusters resulting in impaired isoprenoid biosynthesis. Despite the fact that there are ≈50 iron–sulfur proteins in the genome of *E. coli* [27], deletion of flavodoxin I was not lethal or bacteriostatic as long as exogenous mevalonate was present (Fig. 2B and C). To verify that this phenotype was not the result of a polar effect of the transposon insertion, the *fldA* mutant was complemented with a full-length copy of plasmid-encoded *fldA*. Growth was observed in the *fldA*-complemented mutant in the absence of mevalonate showing that isoprenoid biosynthesis through the MEP pathway was restored (Fig. 2D, G, and H). These results demonstrate that the essential role for flavodoxin I is in the MEP pathway for isoprenoid biosynthesis.

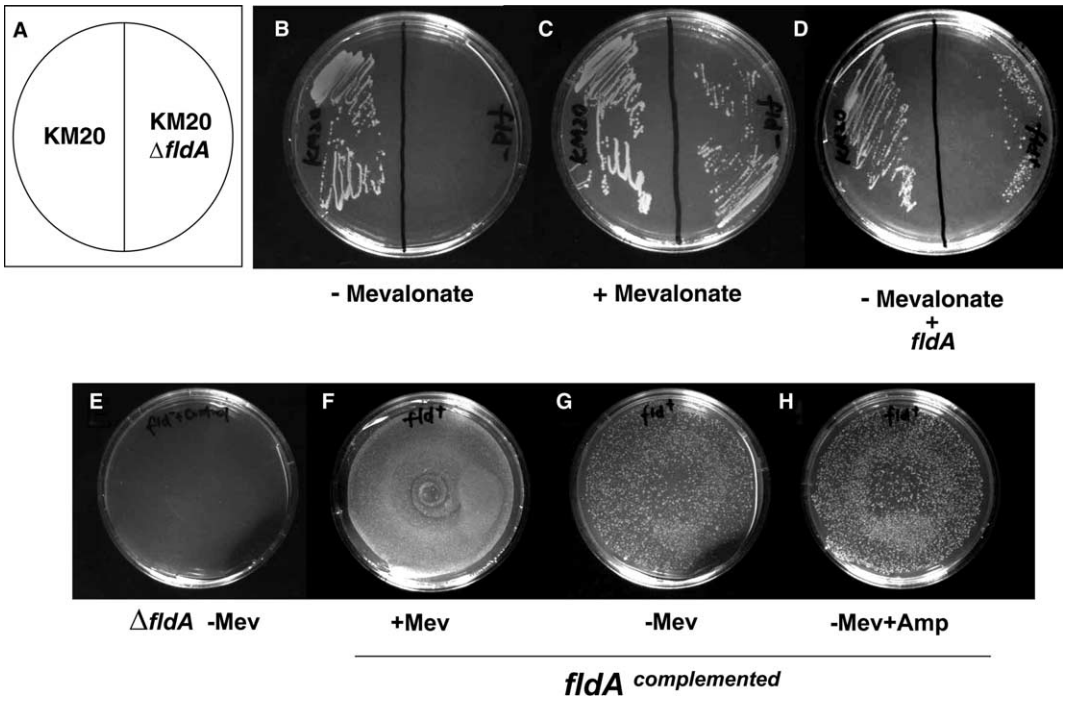

Fig. 2. Mevalonate-independent growth of DK310 LytB^{G120D} (pTMV20KM) is lost after disruption of *fldA* and restored after *fldA* complementation. Panels A–D: Growth of the *E. coli* strains (A) DK310 LytB^{G120D} (pTMV20KM) (termed KM20 on left) and DK310 LytB^{G120D} (pTMV20KM) · *fldA* (termed KM20 · *fldA* on right) on medium (B) without mevalonate, (C) with mevalonate, and (D) without mevalonate after complementation of the KM20 · *fldA* strain with an episomal *fldA* gene. Panels E–H: Growth of KM20 · *fldA* on medium (E) without mevalonate or after complementation with an episomal *fldA* gene (F) with mevalonate, (G) without mevalonate, and (H) without mevalonate but with ampicillin. Note that although complementation of the KM20 · *fldA* strain with an episomal *fldA* gene restored growth without mevalonate, the growth of the complemented bacteria was slower than when growing with mevalonate (compare bacterial growth in panel 2C with panel 2D and panel 2F with panel 2G).

3.3. HMBPP synthesis is fldA-dependent

HMBPP is synthesized by GcpE and is the upstream intermediate for IPP and DMAPP in the MEP pathway. HMBPP is ~30 000-fold more antigenic for human Vγ2Vδ2 T cells stimulation than IPP (data not shown and [11,28]). Synthesis of HMBPP through two one-electron transfers catalyzed by the [4Fe–4S] cluster of the GcpE enzyme has been proposed [9]. Like many [Fe–S] proteins, the enzymatic activity of GcpE depends on the presence of a reducing system. The source of these reducing equivalents is either derived from exogenous reductants (dithiothreitol or deazaflavin) or the native endogenous flavodoxin/flavodoxin reductase/NADPH regeneration system.

In the LytB^{G120D} mutant, DK310 LytB^{G120D} (pTMV20KM), HMBPP was overproduced resulting in a large increase of bioactivity (436-fold) for Vγ2Vδ2 T cell as compared to the wild-type *E. coli* (Fig. 3). This is consistent with previous reports showing that *lytB* deletion blocks the downstream synthesis of IPP and DMAPP causing HMBPP to accumulate [11] and large increases in bioactivity for Vγ2Vδ2 T cells [10]. However, the loss of flavodoxin I in the *fldA*$^{-}$ double mutant led to wild-type levels of bioactivity for Vγ2Vδ2 T cells. Thus, the loss of flavodoxin greatly impairs the ability of GcpE to make HMBPP suggesting that the flavodoxin regeneration system is critical for providing reducing equivalents for the [4Fe–4S] cluster. Although greatly reduced, the bioactivity of the *fldA*$^{-}$ double mutant is still higher than two corresponding gcpE transposon mutants (<500 and 1044 U/L) and a gcpE deletion mutant [29] suggesting that there may be some residual activity of the GcpE enzyme. The LytB enzyme also depends on a [4Fe–4S] cluster for enzymatic activity so the loss of flavodoxin I may also affect LytB function. In summary, the essential role for flavodoxin I is to provide reducing equivalents for the [4Fe–4S] cluster of GcpE and possibly LytB. Both of these enzymes are critical for the growth of *E. coli* under aerobic conditions in rich media.

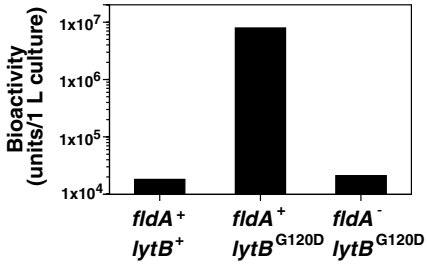

Fig. 3. Loss of flavodoxin I in DK310 LytB^{G120D} (pTMV20KM) decreased HMBPP to the levels of wild-type *E. coli* as assessed by bioactivity for Vγ2Vδ2 T cells. Wild-type *E. coli*, DK310 LytB^{G120D} (pTMV20KM), and DK310 LytB^{G120D} (pTMV20KM) *fldA*$^{-}$ were grown to late stationary phase. Bioactivity for Vγ2Vδ2 T cells was quantitated for bacterial culture supernatants and sonicates using Vγ2Vδ2 T cell clones in an in vitro proliferation assay. Note that the greatly elevated bioactivity of DK310 LytB^{G120D} (pTMV20KM) that have been shown to be due to accumulation of HMBPP [11] was reduced to wild-type levels. Since one unit of bioactivity corresponds to an HMBPP concentration of ~31.6 pM or 31.6 fmol/ml, wild-type bacteria, DK310 LytB^{G120D} (pTMV20KM), and DK310 LytB^{G120D} (pTMV20KM) *fldA*$^{-}$ had bioactivity equivalent to 0.6, 250.3, and 0.7 μmol of HMBPP, respectively.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2005.05.047.

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